

Contents lists available at [ScienceDirect](http://ScienceDirect.com)

Electronic Journal of Biotechnology



Research article

Indole acetic acid and ACC deaminase from endophytic bacteria improves the growth of *Solanum lycopersicum*

Abdul Latif Khan^{a,*}, Boshra Ahmed Halo^a, Ali Elyassi^b, Sajid Ali^{a,c}, Khadija Al-Hosni^a, Javid Hussain^e, Ahmed Al-Harrasi^{a,*}, In-Jung Lee^d

^a UoN Chair of Oman's Medicinal Plants & Marine Natural Products, University of Nizwa, Nizwa, Oman

^b DARIS Center of Research & Development, University of Nizwa, Nizwa, Oman

^c Centre of Biotechnology and Microbiology, University of Peshawar, Pakistan

^d School of Applied Biosciences, Kyungpook National University, Daegu Republic of Korea

^e Department of Biological Sciences & Chemistry, University of Nizwa, Nizwa, Oman

ARTICLE INFO

Article history:

Received 26 November 2015

Accepted 27 January 2016

Available online 18 February 2016

Keywords:

ACC deaminase

Bacillus subtilis LK14

Endophytism

Indole 3-acetic acid

Tomato plant growth

ABSTRACT

Background: Endophytic bacteria are ubiquitous in all plant species contributing in host plant's nutrient uptake and helping the host to improve its growth. *Moringa peregrina* which is a medicinal plant, growing in arid region of Arabia, was assessed for the presence of endophytic bacterial strains.

Results: PCR amplification and sequencing of 16S rRNA of bacterial endophytes revealed the 5 endophytic bacteria, in which 2 strains were from *Sphingomonas* sp.; 2 strains from *Bacillus* sp. and 1 from *Methylobacterium* genus. Among the endophytic bacterial strains, a strain of *Bacillus subtilis* LK14 has shown significant prospects in phosphate solubilization (clearing zone of 56.71 mm after 5 d), ACC deaminase (448.3 ± 2.91 nM α -ketobutyrate $\text{mg}^{-1} \text{h}^{-1}$) and acid phosphatase activity (8.4 ± 1.2 nM $\text{mg}^{-1} \text{min}^{-1}$). The endophytic bacteria were also assessed for their potential to produce indole-3-acetic acid (IAA). Among isolated strains, the initial spectrophotometry analysis showed significantly higher IAA production by *Bacillus subtilis* LK14. The diurnal production of IAA was quantified using multiple reactions monitoring method in UPLC/MS–MS. The analysis showed that LK14 produced the highest (8.7 μM) IAA on 14th d of growth. Looking at LK14 potentials, it was applied to *Solanum lycopersicum*, where it significantly increased the shoot and root biomass and chlorophyll (a and b) contents as compared to control plants.

Conclusion: The study concludes that using endophytic bacterial strains can be bio-prospective for plant growth promotion, which might be an ideal strategy for improving growth of crops in marginal lands.

© 2016 Pontificia Universidad Católica de Valparaíso. Production and hosting by Elsevier B.V. All rights reserved.

1. Introduction

The microbial flora (bacterial and fungal) found in plant species are known as endophytes. These are asymptotically occurring microbes found almost in all parts of living plant species and their organs [1,2,3]. Endophytes are pivotally important for the plant growth and defense mechanisms. Studying the endophytic mode of microbial life and their ecological function has attracted much attention in recent years. The endophytic microbial diversity of beneficial organisms may be more as there are nearly 300,000 terrestrial plant species, whereas each plant can host more than one type of endophyte [4]. Endophytes establish a mutualistic interaction, enhancing host plant's nutrient uptake and helping host to counteract adverse effects of biotic and

abiotic stresses [5]. Colonization of host with such beneficial endophytic bacteria can be used as valuable alternative to synthetic fertilizers to enhance crop productivity [6,7,8].

The beneficial effects of endophytic bacteria or fungi have been regarded for their potential to produce biologically active metabolites and substances [9]. Production of such substances can maintain their own metabolism as well as supports the growth of their hosts [8]. These substances can range from secondary metabolites to various enzymes. In case of metabolites, endophytic microbes have recently been also known to produce plant hormones such as indole 3-acetic acid (IAA), gibberellins and cytokinins etc. [10]. Enzymes include ACC (1-aminocyclopropane-1-carboxylate) deaminase, which are normally related to free-living soil bacteria/rhizobacteria [11,12]. However, for endophytic microbes, this potential has recently been known [11,12,13]. ACC deaminase promotes plant growth by lowering ethylene levels of the plant [10,11,13]. This enzyme catalyzes the conversion of ACC, the immediate precursor of ethylene synthesis in plants, to ammonia and α -ketobutyrate [12]. ACC is exuded from seeds or plant

* Corresponding authors.

E-mail addresses: latifepm78@yahoo.co.uk (A.L. Khan), aharrasi@unizwa.edu.om (A. Al-Harrasi).

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

roots and then metabolized by bacteria possessing ability to produce ACC deaminase. This stimulates plant ACC efflux, decreases the root's ACC and ethylene concentration, thus increases root growth and development [10]. Some of the recent studies have shown that endophytic bacteria can also produce ACC deaminase [12,13], however, this potential has to be explored further.

Endophytes have also been known to produce various classes of secondary metabolites. In case of bioactive metabolites, phytohormones are natural substances mainly produced by plants to regulate its growth [13]. In case of phytohormones, IAA plays a crucial role in plant growth and development, while an additional supply can help the host to safeguard it from environmental stimuli. The production of IAA is widespread among various classes of bacteria/rhizobacteria. Varieties of genus such as *Bacillus*, *Acinetobacter*, *Pseudomonas*, *Azospirillum*, and *Azotobacter* [14] have been reported to produce auxins including IAA. Several different IAA biosynthesis pathways are used by these bacteria, with a single bacterial strain sometimes containing more than one pathway. However, recent studies show that IAA is synthesized through (i) indole-3-pyruvic acid, (ii) indole-3-acetamide and (iii) indole-3-acetonitrile pathways. In the plant growth-promoting bacterium *Azospirillum*, as in *Agrobacterium* and *Pseudomonas*, both the indole-3-pyruvic acid and the indole-3-acetamide pathways are present, as tryptophan (Trp)-dependent and -independent pathways [15].

Recently, plant growth promoting endophytic bacteria (PGPEB) has been found which colonizes plant roots and enhances plant growth by a wide variety of mechanism like phosphate solubilization etc. The use of PGP Rhizobacteria is steadily increased in agriculture and offers an attractive way to replace chemical fertilizers, pesticides, and supplements. Bacteria belonging to the genus *Bacillus* are common rhizospheric microorganisms. These are isolated from various soil samples of various crop plants such as French bean, wheat, soybean, maize, red pepper, rice and lettuce [16]. The previous results support the potential of using *Bacillus subtilis* as biological control of seed pathogens, as well as promoting crop growth [17]. Endophytic mode of *B. subtilis* has been reported recently. It was isolated from the seeds of cacao plant and found greatly contributing to the developmental stages of the cacao plants. Besides this, it has also shown antimicrobial properties [18].

In present study, we isolated various endophytic bacterial strains from the bark of *Moringa peregrina* for the first time. *M. peregrina*, on the other hand, is a medicinal tree growing in arid land with an effective role in abdominal pain, constipation, and headache, and also used as vegetable [19]. Since the host was carrying medicinal importance, therefore, interest was developed to isolate bacterial endophyte from this tree. It was aimed to isolate and characterize the beneficial PGPEB, and to assess its potential in producing IAA and ACC deaminase. To quantify IAA, advance UPLC–MS/MS method was used. It was also aimed to assess whether it can be considered as PGP against crop plants such as tomato.

2. Materials and methods

2.1. Endophyte isolation and identification

The bark of *Moringa peregrina* Forssk. (Moringaceae), growing in the wild mountains of Jabal Al-Akhdar (23° 04' 22.00 N°, 57° 40' 07.00 E"), Sultanate of Oman, was detached from the tree and transported to laboratory in sterile zip bags. To isolate endophytic microbes, a method of Arnold et al. [8] was adopted. Briefly, 20 bark samples were cut into 60 pieces (0.5 cm) and surface sterilized with 2.5% sodium hypochlorite (10 min in a shaking incubator at 120 rpm) and washed with autoclaved distilled water (DW) to remove the contaminants, rhizobacteria and mycorrhizal fungi. The bark pieces were carefully

placed in petri-plates containing Hagem minimal media (0.5% of glucose, 0.05% of KH_2PO_4 , 0.05% of $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.05% of NH_4Cl , 0.1% of FeCl_3 , and 1.5% of agar; pH 5.6 ± 0.2). The sterilized tissues were also imprinted on separate Hagem plates to ensure the effectiveness of the surface sterilization [8].

The bacterial isolates were identified on the basis of partial 16S ribosomal rRNA sequence. The 16S rRNA was amplified by PCR using the 27F primer (5'-AGAGTTTGATC(AC)TGGCTCAG-3') and 1492R primer (5'-CGG(CT)TACCTTGTACGACTT-3'), which were complementary to the 5' end and 3' end of the prokaryotic 16S rRNA, respectively. The amplification reaction was performed as previously described. The BLASTn search programs (<http://www.ncbi.nlm.nih.gov/BLASTn>) were used to compare the sequence homology of nucleotides. The closely related sequences obtained were aligned through CLUSTALW using MEGA version 4.0 software [19] and the maximum parsimony tree was constructed using the same software. The bootstrap replications (1 K) were used as a statistical support for the nodes in the phylogenetic tree.

2.2. Screening for IAA production

Estimation of IAA in the culture broth was done using colorimetric assay [20]. The endophytes cultured in 20 mL of LB broth without (0 g/L) and with 0.1 g/L of L-tryptophan and incubated at $30 \pm 2^\circ\text{C}$ in shaking incubator at 200 rpm for 7 d. The bacterial cultures were then centrifuged at $10,000 \times g$ for 10 min at 4°C and the cell free cultures were filtered through 0.45 μm cellulose acetate filter (DISMIC®, Denmark). The filtrates were acidified to pH 2.8 with 1 N of HCl and extracted 3 times with 20 mL of ethyl acetate. The ethyl acetate fractions were combined and evaporated under vacuum at 45°C in a rotary evaporator. The residue was re-suspended in 3 mL 50% methanol. One milliliter of supernatant was mixed with 2 mL of Salkowski reagent (12 g of FeCl_3/L , 7.9 M of H_2SO_4) and kept in dark for 30 min. The resultant reddish color was read after 30 min at 535 nm in ELISA Spectrophotometer (BioRad, USA). The amount of IAA was calculated with standard of pure IAA (Sigma-Aldrich Ltd, Korea,) prepared separately.

2.3. IAA quantification by UPLC/MS MS

After finding positive results of endophyte, we selected one strain which was showing highest IAA producing ability. Bacterial IAA was quantified using Waters Ultra Performance Liquid Chromatography (UPLC) system (Acquity UPLC, Waters Inc. USA). The analytical column used was Waters Acquity BEH HILIC ($2.1 \times 50 \text{ mm } 1.7 \mu$) and the column oven temperature was set to 40°C . The injection volume was set at 10 μL and the injection mode was partial loop with needle overfill (PLUNO). The flow rate was set at 0.2 mL/min using channel A, 0.5% of formic acid in water; and channel B, 0.5% of formic acid in acetonitrile. The mobile phase was run on a gradient profile with a starting point of 95% A. This was held for 3 min then changed to 10% and held for 1 min. At 4.1 min the starting composition was introduced. With the pre-equilibration time of 1.9 min the total analysis time was 6 min.

The Acquity UPLC was interfaced with a Waters Quattro Premier XE Tandem Quadrupole system (Waters Inc., USA). The tuning parameters were optimized using a standard solution of approximately 4 ng/ μL (ppm) of IAA. For these measurements, electrospray ionization (ESI+) was used with the source operation parameters set as follows: Capillary voltage: 3.0 kV, Cone voltage: 12.0 V, Collision Energy: 13 eV, source temperature: 150°C and desolvation temperature: 400°C . The desolvation gas was set at 1000 L/h and cone gas at 20 L/h. The Precursor ion was optimized at 175.65 and the Product ion 129.8.

2.4. Verification and quantification of IAA by LC–MS/MS

For this experiment the IAA standard was dissolved in 100% water. The weak reverse phase solvent as well as the gradient mobile phase helped to produce good peak shape. The quantification of IAA was carried out using two external standard preparation and average response factor for the two standards. This was done by integrating the area under the peak for each Multiple Reaction Monitoring (MRM) trace which was obtained from transition of Precursor ion 175.65 to 129.8 Product ion for both the standards and the samples. The purity of the standard (98% w/w) was also taken into consideration during the response factor calculations.

2.5. Phosphate solubilization and acid phosphate activity

To detect the phosphate solubilizing bacteria, strains were streaked onto Pikovskaya's agar medium, which contains (per liter): 0.5 g of yeast extract, 10 g of dextrose, 5 g of $\text{Ca}_3(\text{PO}_4)_2$, 0.5 g of $(\text{NH}_4)_2\text{SO}_4$, 0.2 g of KCl, 0.1 g of $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.0001 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.0001 g of $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ and 15 g of agar. After 3 d of incubation at 28°C, strains that induced clear zone around the colonies were considered as positive [20]. Strains were collected from the cultures and immediately resuspended in water. This suspension was centrifuged ($5000 \times g$, 5 min at 4°C) and the pellet was washed twice in water and once in 50 mM of sodium acetate buffer, pH 5.5, before being re-suspended in the same buffer. The preparation was then mixed with glass beads in an ice bath and fragmented in a vortex mixer at maximum speed for 30 s, followed by immediate cooling in an ice bath for 2 min (this procedure was repeated five times). The glass beads were removed by sedimentation and the supernatant was centrifuged ($5000 \times g$, 5 min, 4°C) to remove unbroken cells and cell debris. The supernatant was collected for enzyme analysis.

Acid phosphatase activity was assayed by measuring *p*-nitrophenylphosphate (*p*NPP) hydrolysis [21] the assay mixture contained sodium acetate buffer (50 mM, pH 5.5), 3 mM of *p*NPP and 5–10 µg of protein from the homogenate, in a final volume of 325 µL. After 10 min at 37°C, the reaction was stopped by adding 1 mL of 0.2 M NaOH. The activity was expressed in nano moles of *p*-nitrophenol (*p*NP) formed/mg of protein/min, based on an extinction coefficient of $18,400 \text{ M}^{-1} \text{ cm}^{-1}$ at 410 nm.

2.6. ACC deaminase activity

ACC deaminase activity was assayed according to a modified method of [22,23] which measures the amount of α -ketobutyrate produced upon the hydrolysis of ACC. The number of µmol of α -ketobutyrate produced by this reaction was determined by comparing the absorbance at 540 nm of a sample to a standard curve of α -ketobutyrate ranging between 10 and 200 µmol. A stock solution of 100 mmol L^{-1} α -ketobutyrate was prepared in 0.1 mol L^{-1} of Tris–HCl, (pH 8.5) and stored at 4°C. Just prior to use, the stock solution was diluted with the same buffer to make 10 mmol L^{-1} of solution from which a standard concentrations curve was generated. In a series of known α -ketobutyrate concentrations, 2 mL of the 2,4-dinitrophenyl-hydrazine reagent (0.2% 2, 4-dinitrophenyl-hydrazine in 2 mol L^{-1} HCl) was added, the contents were vortexed and incubated at 30°C for 30 min, during which the α -ketobutyrate was derivitized as aphenylhydrazine. The color of phenyl hydrazine was developed by the addition of 2 mL, 2 mol L^{-1} of NaOH, the absorbance of the mixture was measured after mixing by using spectrophotometer at 540 nm.

For determining ACC deaminase activity, endophytes strains were grown in rich Tryptic Soy Broth medium (TSB) for 18 h at 28°C. The cells were then harvested by centrifugation, washed with 0.1 M Tris–HCl (pH 7.5), and incubated for another 18 h in

minimal medium containing 3 mM ACC as the sole source of nitrogen. The bacterial cells were collected by centrifugation [20] and suspended in 5 mL of 0.1 mol L^{-1} of Tris–HCl, pH 7.6, and transferred to microcentrifuge tube. The contents of the tubes were centrifuged at 16,000 rpm for 5 min and supernatant was removed. The pellets were suspended in 2 mL 0.1 mol L^{-1} Tris HCl, pH 8.5. Thirty microliters of toluene was added to the cell suspension and vortexed for 30 s. 200 µL of the toluenized cells were placed in a fresh microcentrifuge tube, 20 µL of 0.5 mol L^{-1} ACC was added to the suspension, vortexed, and then incubated at 30°C for 15 min, following the addition of 1 mL of 0.56 mol L^{-1} HCl, the mixture was vortexed and centrifuged for 5 min at 13,000 rpm at room temperature. 2 mL of the supernatant was vortexed together with 1 mL of 0.56 mol L^{-1} HCl. Thereupon, 2 mL of the 2,4-dinitrophenylhydrazine reagent (0.2% 2, 4-dinitrophenylhydrazine in 2 mol L^{-1} HCl) was added to the glass tube, and the contents were vortexed and then incubated at 30°C for 30 min. Following the addition and mixing of 2 mL of 2 mol L^{-1} of NaOH, the absorbance of the mixture was measured by using spectrophotometer at 540 nm [23]. The cell suspension without ACC was used as negative control and with $(\text{NH}_4)_2\text{SO}_4$ (0.2% w/v) as positive control.

2.7. Effect of endophyte inoculation on tomato plant growth

Among endophytic bacterial strains, only *B. subtilis* showed highest IAA production capacity. Therefore, to assess its potential in plant growth regulation, the bacterial strain was applied on tomato plants. Briefly, bacteria culture suspension was incubated for 3 d at 30°C in a shaking incubator at 200 rpm to an estimated cell density of 10^8 CFU/mL. Tomato (*Solanum lycopersicum* cv.) seeds (Seminis Korea Co. Korea) were surface sterilized with NaOCl (5%) for 10 min and thoroughly rinsed with autoclaved distilled water. Seeds were sown in autoclaved soil (peat moss (12–18%), perlite (8–12%), coco-peat (62–68%) and zeolite (6–8%)), while the macro-nutrients present were as follows: NH_4^+ ~90 mg/L, NO_3^- ~205 mg/L, P_2O_5 ~350 mg/L, and K_2O ~100 mg/L) plastic pots under controlled greenhouse conditions at $28 \pm 2^\circ\text{C}$. Tomato seedlings were treated with 5 mL of bacterial suspension 2 weeks after sowing and the growth attributes i.e. shoot length, shoot and root fresh weight, shoot and root dry weight, and chlorophyll contents were recorded after 21 d of treatment. The experiment comprised three treatments and four replicates. Each replicate consists of 24 plants. Distilled water and NB media were used as negative and positive controls respectively during the experiment. Photosynthetic pigments were extracted from leaves of Tomato plants ground with 80% acetone. The chlorophylls and carotenoid were estimated according to the method of Lichtenthaler. The absorbance for chlorophylls a and b and carotenoid was recorded at 663, 645, and 470 nm, respectively. Chlorophyll content was calculated using the following formulae:

$$\text{Chlorophyll a (mg/g FW)} = \frac{[(12.7 \times A_{663}) - (2.69 \times A_{645})]}{1000 \times W} \times V$$

$$\text{Chlorophyll b (mg/g FW)} = \frac{[(22.9 \times A_{645}) - (4.68 \times A_{663})]}{1000 \times W} \times V$$

where *W* is the fresh weight and *V* is the extraction volume [19].

2.8. Statistical analysis

The data were analyzed statistically for standard deviation and error by using GraphPad Prism (Ver 5.0; CA USA). The mean values were compared using Duncan's multiple range tests at $p < 0.05$ (SAS, Cary, NC, USA).

3. Results and discussion

3.1. Isolation of endophytic bacteria from the bark of *M. peregrina*

We isolated five endophytic bacterial strains from the bark of *M. peregrina*. The isolates were grouped upon morphological analysis such colony shape, size, color, pattern and texture. The five isolates were subjected to DNA extraction, PCR amplification, sequencing and phylogenetic analysis using universal primers for 16S rRNA region. Phylogenetic analysis of the bacterial strain was performed and a neighbor joining (NJ) tree was made using MEGA 6.0. Consensus tree were constructed using reference and homologues sequences aligned with 16S rRNA sequence (statistical support of 1000 bootstrap replications; Supplementary information). Results of BLASTn search (National Center of Biotechnology Information – NCBI) revealed that the sequences of isolated endophytic bacteria from *M. peregrina* had ~90% sequence homology. The results showed that among 5 endophytic bacteria, 2 strains were from *Sphingomonas* sp.; 2 strains from *Bacillus* and 1 from *Methylobacterium* genus (Table 1). The sequence data was submitted to Gen-Bank with the accession number. Previously, there are a few endophytic microbes isolated from *M. peregrina*. Among reported endophytic micro flora *Aspergillus caespitosus* LK12 and *Phoma* sp. LK13 [19], however, there is no report of endophytic bacterial diversity from this medicinal tree.

3.2. ACC deaminase production by endophytic bacteria

Enzymes such as ACC (1-aminocyclopropane-1-carboxylate) deaminase are produced by the various bacterial strains and its production is mostly related to free-living soil bacteria and some fungi. However, more recently, it has been reported from rhizobacteria and endophytic bacteria [10,11,12,13]. ACC deaminase-producing bacteria promote plant growth under stress conditions and also playing a very important role in the process of nodulation, specific to plant species. According to Onofre-Lemus et al. [24] the bacterial enzyme ACC (1-aminocyclopropane-1-carboxylate) deaminase promotes plant growth by lowering plant ethylene levels. ACC deaminase was observed in PGPEB strains by their growth on Dworking and Foster (DF) minimal medium containing ACC. MPB2.1 strain had the highest value of deamination of ACC (454 nmol α -ketobutyrate $\text{mg}^{-1} \text{h}^{-1}$) followed by MPB2.1, MPS21, MPB4.1, MPS1.2 and MPB6.1 strain. In this way, MPB6.1 strain had the lowest value (310 nmol α -ketobutyrate $\text{mg}^{-1} \text{h}^{-1}$) in ACC deaminase activity (Fig. 1). Similarly, other three strains MPB4.1, MPS21 and MPS1.2 also showed significantly different value for ACC deaminase activity. The growth promoting activity of ACC deaminase from *Pseudomonas fluorescens* strains on groundnut seedlings showed a value of 342 nmol α -ketobutyrate $\text{mg}^{-1} \text{h}^{-1}$ [12]. Moreover, it has been reported that some ACC deaminase-producing bacteria promote plant growth under abiotic stress conditions [25,26,27]. In addition, this enzyme has a pivotal role in the process of nodulation in different plant species [27].

Table 1

Endophytic bacteria isolated and identified from the bark of *M. peregrina* and their potential to produce IAA using spectrophotometer.

Code name	Endophytic bacteria	μM in 50 mL broth
MPB 4.1	<i>Sphingomonas</i> sp. LK18	$35.19 \pm 0.38\text{b}$
MPS 21	<i>Methylobacterium radiotolerans</i> LK17	$31.13 \pm 0.48\text{c}$
MPB 2.1	<i>B. subtilis</i> LK14	$165.53 \pm 1.01\text{a}$
MPB 6.1	<i>B. subtilis</i> LK15	$32.01 \pm 0.81\text{c}$
MPS 1.2	<i>Sphingomonas</i> sp. LK16	$25.46 \pm 0.13\text{cd}$

± shows the standard error of three replications. Different letters in the column show that values are significantly different ($p < 0.05$) from each other as evaluated from DMRT test.

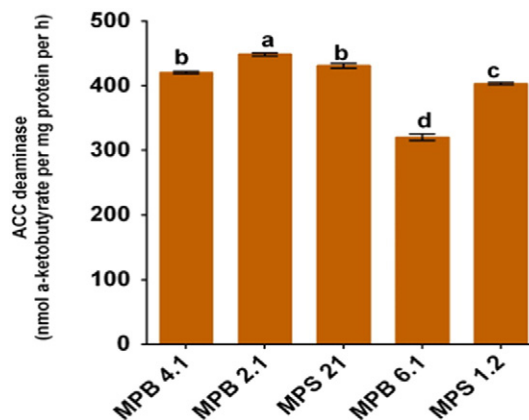


Fig. 1. ACC deaminase by the isolated endophytes in (DF) minimal medium containing ACC. MPB2.1 strain had the highest value in ACC deaminase. Different letters in the column show that values are significantly different ($p < 0.05$) from each other as evaluated from DMRT (Duncan's Multiple Range Test) test.

3.3. Phosphate solubilization and acid phosphatase potentials of Endophyte

The isolated endophytic culture filtrates from *M. peregrina* were screened for phosphate solubilization and acid phosphatase potentials. The screening results showed that phosphate solubilization by MPB 2.1 strain produced a clear zone around the colonies which shows the highest phosphatase solubilization potential on NBRIP medium. Various researchers have documented acid phosphatase activity in the different strains of *Bacillus* species. The previous study showed that *Enterobacter asburiae* and *Bacillus cereus* enhanced phosphate solubilization ($165\text{--}415 \text{ g L}^{-1}$) upon 4 d of growth [17,18]. In our results the growth of MPB 2.1 showed the phosphate clearing zone of 56.716 mm after 5 d of growth. Similarly the research work of Almoneafy et al. [28] showed that three strains of *Subtilus* D29, Am1 and H8 have capability to solubilize phosphate. The results of Rodríguez and Fraga [25] showed that *Pseudomonas striata* and *Bacillus polymyxa* solubilized 156 and 116 mg P L^{-1} , respectively. In our results MPB 2.1 strain showed prominent results for phosphate solubilization ($8.4 \text{ nM mg}^{-1} \text{Min}^{-1}$) followed by MPS1.2 ($6.3 \text{ nM mg}^{-1} \text{Min}^{-1}$) and MPB6.1 ($5.9 \text{ nM mg}^{-1} \text{Min}^{-1}$) Fig. 2. In this way the present results are in conformity with the previous data of phosphate solubilization.

3.4. IAA quantification from endophytic bacteria

Plants regulate its developmental process by producing phytohormones in its various parts, while endophytic microbial flora

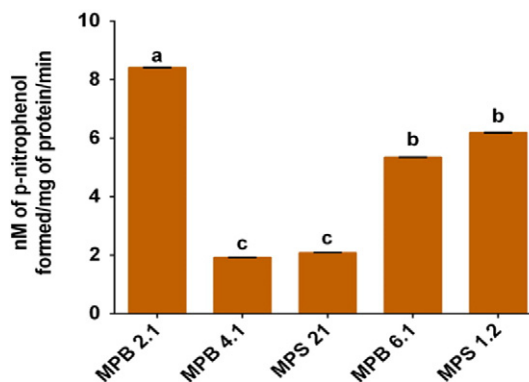


Fig. 2. Acid phosphatase activity of different endophytic bacterial strains of *M. peregrina*. Different letters in the column show that values are significantly different ($p < 0.05$) from each other as evaluated from DMRT test.

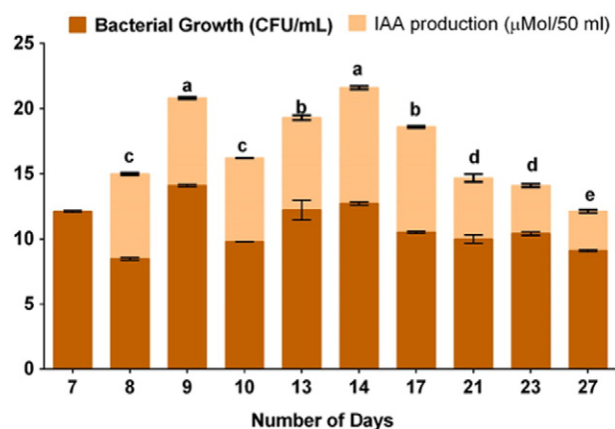


Fig. 3. Endophytic MPB 1.2 (*B. subtilis* LK14) growth over a period of time and production of IAA. Different letters show that values are significantly different ($p < 0.05$) from each other as evaluated from DMRT test.

also contributing in the production of these phytohormones. It is now a matter-of-fact that IAA can be produced by variety of endophytes [9,15]. Similarly, in our study endophytic bacteria *B. subtilis* LK14 was isolated from *M. peregrina*, and were applied on tomato plants. As IAA plays a vital role in plant development and at the same time its additional supply can support the host in stress conditions such drought [22] and pathogenic attacks [14].

There are various types of rhizospheric bacteria which are documented for the production of IAA, exploit different IAA biosynthesis pathways and single bacterial strain sometimes encompassing more than one pathway [14,15]. With reference to earlier data, IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability [1]. In our results the MPB 2.1 strain of *B. subtilis* LK14 showed the highest results (165.53 μM in 50 mL culture broth) for IAA production, while *B. subtilis* LK15 produced a little amount (32.01 μM in 50 mL culture broth) as compared to *B. subtilis* LK14 (Fig. 3; Table 1). Similarly, the study of Fuentes-Ramirez et al. [19] showed that various strains of *Acetobacter diazotrophicus* produced IAA in defined culture medium and their HPLC analyses revealed that *A. diazotrophicus* strains produced from 0.14 to 2.42 μg IAA mL⁻¹ in culture medium.

In our studies, for the estimation of IAA in culture broth sophisticated methodologies were used as Colorimetric assay in addition with L-tryptophan which favored the high amount of IAA. Then PGPEB IAA was quantified by using Waters UPLC system and further verification and quantification of IAA was brought about by LC-MS/MS. Our results of mass spectroscopy showed that the strains of *B. subtilis* LK14 give a high value of IAA on 9th day in their culture medium which is 165.53 mMol/50 mL LB broth (Fig. 4). The greater amount of IAA produced by PGPEB in LB broth is because of L-tryptophan, which is also confirmed from the results of Idris et al. [5] revealed that IAA secretion can be increased by providing tryptophan. In this way, the Inoculation of IAA producing bacteria

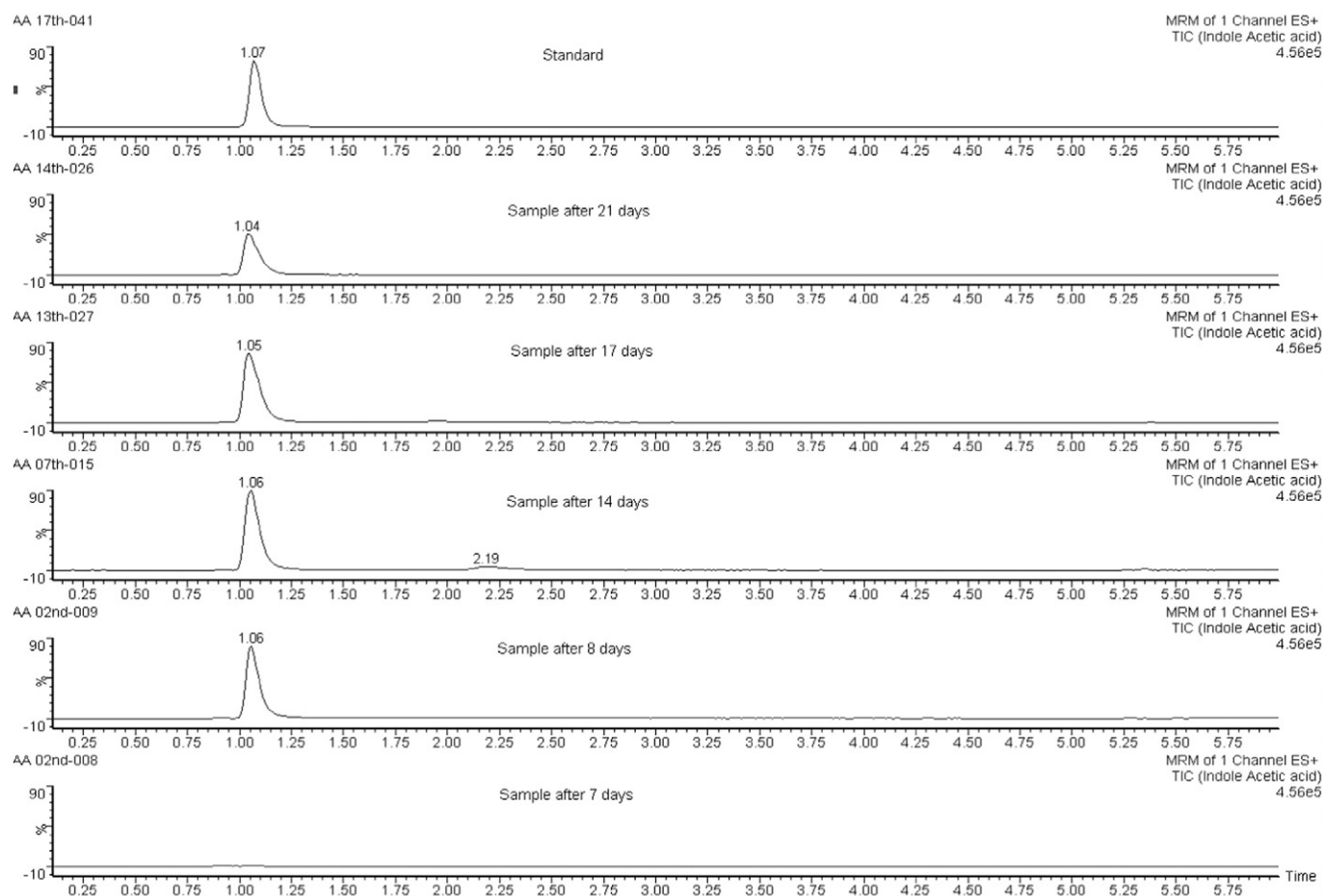


Fig. 4. UPLC-MS/MS analyses of culture filtrate (CF) of MPB 2.1 (*B. subtilis* LK14) during different days of growth. The CF analysis showed that IAA content was absent for the first 7 d. After continuously growing the strain, it showed significantly higher IAA content on 14th d of incubation. The chromatogram shows the IAA peaks after 8, 14, 17 and 21 d of inoculation. The results were obtained by integrating the peak area for each MRM trace which was obtained from transition of Precursor ion 175.65 to 129.8 product ions for both the standards and the samples.

induce the development of tomato plants. In the present study IAA production PGPEB are in agreement with previous reports.

The two different endophytic species of *Sphingomonas* LK18 and LK16 produce 35.19 and 25.46 μM in 50 mL broth respectively while, *Pseudomonas aeruginosa* LK17 encodes for 31.13 μM in 50 mL culture broth. By comparing the IAA amount produced by endophytic *B. subtilis* LK14 and *B. subtilis* LK15, the former strain produce 52% greater amount of IAA in LB broth medium.

While the results of mass spectroscopy revealed that some strains of *B. subtilis* give a high value on 9th d in their culture medium which is 14.02 $\mu\text{Mol}/50\text{ mL}$ (Fig. 4). According to Srinivasan et al. [27] the different strains of *Bacillus* showed significant difference in the amount of IAA varying from 0.40 to 4.88 $\mu\text{g}/\text{mL}$, for the quantification of IAA they used thin-layer chromatography, high-performance liquid chromatography, gas chromatography, mass spectrometry, and the Avena Coleoptile bioassay. In our results the greater amount of IAA produced by these endophytes in 20 mL LB broth is because of L-tryptophan which was incubated at $30 \pm 2^\circ\text{C}$ in shaking incubator at 200 rpm for one week. Auxin production by *Bacillus* spp. in L-broth medium supplemented with 1000 $\mu\text{g mL}^{-1}$ L-tryptophan ranges from 0.60 to 3.0 $\mu\text{g IAA mL}^{-1}$ as revealed by gas chromatography and mass spectrometric (GC-MS) analysis [1]. While the results of Idris et al. [5] also showed that IAA secretion can be increased up to fivefold by providing 5 mM of tryptophan in the culture medium.

3.5. Effect on the tomato plant growth

The screening results showed that application of endophytic bacterial strain MPB 2.1 culture significantly enhanced the development of tomato seedling. The Shoot length, Shoot weight, Root length, and chlorophyll contents were significantly boosted by the application of MPB 2.1 culture (Fig. 5a). The length of shoot and root were paralleled with control and it is deciphered from the results that the tomato plantlet treated with endophytic bacterial strain MPB 2.1 showed upsurge in length 2.5 cm and 3.5 cm respectively. Almoneafy et al. [28] noted a positive correlation (0.777 and 0.686) between the IAA amounts produced by *Bacillus* strains of bacterized tomato plants inside the greenhouse which showed that these bacterial strains are promoting the growth of tomato plants. Additionally, this was the first report which demonstrated the mode of antibacterial effect of *Bacillus* strains against *R. solanacearum*.

Similarly, in our results the strain MBP 2.1 produced a significant effect on the weight of both these parts. The weight of shoot was recorded as 400 mg greater as compare to control, while the weight of root was 450 mg more than the weight of control (Fig. 5b). The strain JH 2-2 of *Bacillus* species, which were isolated from the rhizosphere of plants at a multi-metal contaminated mine site, has the potential to reduce Cr(VI) to Cr(III) and promote plant growth by reducing Cr toxicity and producing IAA in stress condition.

Chlorophylls (a and b) and carotenoid were recorded at 663, 645, and 470 nm, respectively. As tomato seedlings were treated with bacterial suspension and chlorophyll contents were recorded after three weeks of the treatment. The chlorophyll contents were significantly enhanced by the application of MPB 2.1 culture after 9th d. By comparing the results chlorophyll a contents showed an increase of 0.4 mg/g while 0.34 mg/g in chlorophyll b contents were recorded. In this way, the total carotenoid presented the pragmatic results with comparison to control (Fig. 5c).

In conclusion, endophytes are the microbial flora which are residing asymptotically in all plants and enhancing host plant's nutrient uptake and helping host to counteract adverse effects of biotic and abiotic stresses by producing a variety of secondary metabolites. In present study various endophytic bacterial strains were isolated from the bark of *M. peregrine* and were evaluated for their potential in producing IAA, phosphate solubilization and ACC deaminase activity. Advance and sophisticated strategies were used for the evaluation of

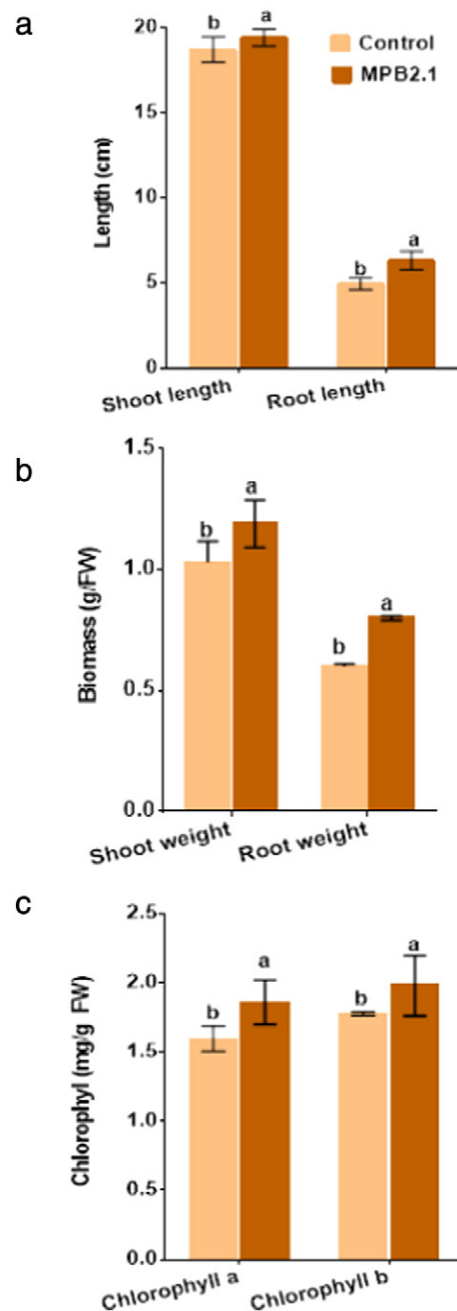


Fig. 5. Effect of *B. subtilis* LK14 (MPB 2.1) on a: the plant length; b: plant biomass; and c: chlorophyll contents of tomato plants. Different letters show that values are significantly different ($p < 0.05$) from each other as evaluated from DMRT test.

all these activities. And finally, the plant growth promoting effects of these isolated strains were tested on tomato plants. Among all tested strains the endophytic *B. subtilis* LK14 showed prominent results and is capable to be utilized as PGP in crop plants.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ejbt.2016.02.001>.

Financial support

The authors acknowledge the financial support of the Oman Research Council (TRC) through Open Research Grant #ORG/CBS/12/004 and University of Nizwa Research Fund (A/13-14-UoN/01/Chair MPMNP/IF).

Conflict of interest

The authors declare no conflict of interest.

References

- [1] Fíráková S, Šturdíková M, Múčková M. Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia* 2007;62:251–7. <http://dx.doi.org/10.2478/s11756-007-0044-1>.
- [2] Arendt KR, Baltrus DA, Arnold AE. Diversity and specificity of phenotypic effects of endohyphal bacteria on foliar fungal endophytes. *Phytopathology* 2013;103:S2.
- [3] Limsuwan S, Trip EN, Kouwen TRHM, Piersma S, Hiranrat A, Mahabusekaram W, et al. Rhodomyrtone: A new candidate as natural antibacterial drug from *Rhodomyrtus tomentosa*. *Phytomedicine* 2009;16:645–51. <http://dx.doi.org/10.1016/j.phymed.2009.01.010>.
- [4] Strobel G, Daisy B, Castillo U, Harper J. Natural products from endophytic microorganisms. *J Nat Prod* 2004;67:257–68. <http://dx.doi.org/10.1021/np030397v>.
- [5] Idris EE, Iglesias DJ, Talon M, Borriss R. Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. *Mol Plant Microbe Interact* 2007;20:619–26. <http://dx.doi.org/10.1094/MPMI-20-6-0619>.
- [6] Dutta D, Puzari KC, Gogoi R, Dutta P. Endophytes: Exploitation as a tool in plant protection. *Braz Arch Biol Technol* 2014;57:621–9. <http://dx.doi.org/10.1590/S1516-8913201402043>.
- [7] Farrar K, Bryant D, Cope-Selby N. Understanding and engineering beneficial plant-microbe interactions: Plant growth promotion in energy crops. *Plant Biotechnol J* 2014;12:1193–206. <http://dx.doi.org/10.1111/pbi.12279>.
- [8] Arnold AE, Henk DA, Eells RL, Lutzoni F, Vilgalys R. Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. *Mycologia* 2007;99:185–206. <http://dx.doi.org/10.3852/mycologia.99.2.185>.
- [9] Saxena S. Microbial metabolites for development of ecofriendly agrochemical. *Allelopathy J* 2014;33:1–24.
- [10] Hardoim PR, van Overbeek LS, Elsas JD. Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol* 2008;16:463–71. <http://dx.doi.org/10.1016/j.tim.2008.07.008>.
- [11] Glick BR. Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol Res* 2014;169:30–9. <http://dx.doi.org/10.1016/j.micres.2013.09.009>.
- [12] Glick BR, Todorovic B, Czarny J, Cheng Z, Duan J, McConkey B. Promotion of plant growth by bacterial ACC deaminase. *Crit Rev Plant Sci* 2007;26:227–42. <http://dx.doi.org/10.1080/07352680701572966>.
- [13] Rashid S, Charles TC, Glick BR. Isolation and characterization of new plant growth-promoting bacterial endophytes. *Appl Soil Ecol* 2012;61:217–24. <http://dx.doi.org/10.1016/j.apsoil.2011.09.011>.
- [14] Cassán F, Vanderleyden J, Spaepen S. Physiological and agronomical aspects of phytohormone production by model plant-growth-promoting rhizobacteria (PGPR) belonging to the genus *Azospirillum*. *Plant Growth Regul* 2014;33:440–59. <http://dx.doi.org/10.1007/s00344-013-9362-4>.
- [15] Duca D, Lorv J, Patten CL, Rose D, Glick BR. Indole-3-acetic acid in plant-microbe interactions. *Antonie Van Leeuwenhoek* 2014;106:85–125. <http://dx.doi.org/10.1007/s10482-013-0095-y>.
- [16] Park M, Kim C, Yang J, Lee H, Shin W, Kim S, et al. Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. *Microbiol Res* 2005;160:127–33. <http://dx.doi.org/10.1016/j.micres.2004.10.003>.
- [17] Chen F, Wang M, Zheng Y, Luo J, Yang X, Wang X. Quantitative changes of plant defense enzymes and phytohormone in biocontrol of cucumber Fusarium wilt by *Bacillus subtilis* B579. *World J Microbiol Biotechnol* 2010;26:675–84. <http://dx.doi.org/10.1007/s11274-009-0222-0>.
- [18] Araujo FF, Henning AA, Hungria M. Phytohormones and antibiotics produced by *Bacillus subtilis* and their effects on seed pathogenic fungi and on soybean root development. *World J Microbiol Biotechnol* 2005;21:1639–45. <http://dx.doi.org/10.1007/s11274-005-3621-x>.
- [19] Khan AL, Waqas M, Hussain J, Al-Harrasi A, Al-Rawahi A, Al-Hosni K, et al. Endophytes *Aspergillus caespitosus* LK12 and *Phoma* sp. LK13 of *Moringa peregrina* produce gibberellins and improve rice plant growth. *J Plant Interact* 2014;9:731–7. <http://dx.doi.org/10.1080/17429145.2014.917384>.
- [20] Ullah I, Khan AR, Park GS, Lim JH, Waqas M, Lee IJ, et al. Analysis of phytohormones and phosphate solubilization in *Photobacterium* spp. *Food Sci Biotechnol* 2013;22:25–31. <http://dx.doi.org/10.1007/s10068-013-0044-6>.
- [21] Galabova DN, Tuleva B, Balasheva MA. A rapid method for determination of acid phosphatase activity of whole yeast cells. *Lett Appl Microbiol* 1993;16:161–3. <http://dx.doi.org/10.1111/j.1472-765X.1993.tb01384.x>.
- [22] Belimov AA, Dodd IC, Safronova VI, Shaposhnikov AI, Azarova TS, Makarova NM, et al. Rhizobacteria that produce auxins and contain 1-amino-cyclopropane-1-carboxylic acid deaminase decrease amino acid concentrations in the rhizosphere and improve growth and yield of well-watered and water-limited potato (*Solanum tuberosum*). *Ann Appl Biol* 2015;167:11–25. <http://dx.doi.org/10.1111/aab.12203>.
- [23] Shaharoon B, Riffat B, Muhammad A, Zahir Z, Ul-Hassan Z. 1-Aminocyclopropane-1-carboxylate (acc)-deaminase rhizobacteria attenuates acc-induced classical triple response in etiolated pea seedlings. *Pak J Bot* 2006;38:1491–9.
- [24] Onofre-Lemus J, Hernández-Lucas I, Girard L, Caballero-Mellado J. ACC (1-aminocyclopropane-1-carboxylate) deaminase activity, a widespread trait in *Burkholderia* species, and its growth-promoting effect on tomato plants. *Appl Environ Microbiol* 2009;75:6581–90. <http://dx.doi.org/10.1128/AEM.01240-09>.
- [25] Rodríguez H, Fraga R. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol Adv* 1999;17:319–39. [http://dx.doi.org/10.1016/S0734-9750\(99\)00014-2](http://dx.doi.org/10.1016/S0734-9750(99)00014-2).
- [26] Fuentes-Ramírez L, Jiménez-Salgado T, Abarca-Ocampo IR, Caballero-Mellado J. *Acetobacter diazotrophicus*, an indoleacetic acid producing bacterium isolated from sugarcane cultivars of Mexico. *Plan Soil* 1993;154:145–50. <http://dx.doi.org/10.1007/BF00012519>.
- [27] Srinivasan M, Holl FB, Petersen DJ. Influence of Indole acetic acid producing *Bacillus* isolates on the nodulation of *Phaseolus vulgaris* by *Rhizobium etli* under gnotobiotic conditions. *Can J Microbiol* 1996;42:1006–14. <http://dx.doi.org/10.1139/m96-129>.
- [28] Almoneafy AA, Xie GL, Tian WX, Xu LH, Zhang GQ, Ibrahim M. Characterization and evaluation of *Bacillus* isolates for their potential plant growth and biocontrol activities against tomato bacterial wilt. *Af J Biotech* 2014;11(28):7193–201.